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J Guastella, N Brecha, C Weigmann, HA Lester, and N Davidson

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Cloning, expression, and localization of a rat brain high-affinity glycine transporter

JOHN GUASTELLA*, NICHOLAS BRECHA†, CHRISTINE WEIGMANN†, HENRY A. LESTER*,
AND NORMAN DAVIDSON*

*Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125; and †Departments of Anatomy and Cell Biology and Medicine, Brain Research Institute and Center for Ulcer Research, University of California, Los Angeles School of Medicine and Veterans Administration Medical Center–Wadsworth, Los Angeles, CA 90073

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ABSTRACT A cDNA clone encoding a glycine transporter has been isolated from rat brain by a combined PCR and plaque-hybridization strategy. mRNA synthesized from this clone (designated GLYT1) directs the expression of sodium- and chloride-dependent, high-affinity uptake of [³H]glycine by *Xenopus* oocytes. [³H]Glycine transport mediated by clone GLYT1 is blocked by sarcosine but is not blocked by methyl-aminoisobutyric acid or L-alanine, a substrate specificity similar to that described for a previously identified glycine-uptake system called system Gly. *In situ* hybridization reveals that GLYT1 is prominently expressed in the cervical spinal cord and brainstem, two regions of the central nervous system where glycine is a putative neurotransmitter. GLYT1 is also strongly expressed in the cerebellum and olfactory bulb and is expressed at lower levels in other brain regions. The open reading frame of the GLYT1 cDNA predicts a protein containing 633 amino acids with a molecular mass of ≈70 kDa. The primary structure and hydropathicity profile of GLYT1 protein reveal that this protein is a member of the sodium- and chloride-dependent superfamily of transporters that utilize neurotransmitters and related substances as substrates.

Termination of synaptic activity is thought to occur through removal of neurotransmitter from the synaptic cleft by ion-coupled, high-affinity neurotransmitter transport proteins (neurotransmitter transporters) located in neuronal and glial plasma membranes. Drugs that block the action of these transporters can modulate neural function, probably by increasing the duration of neurotransmitter action. Some neurotransmitter transporters, such as those for the monoamines, are the sites of action for clinically important drugs (for example, antidepressants) (1), as well as drugs of abuse (for example, cocaine) (2). Other neurotransmitter transporters, such as those for the inhibitory amino acid γ -aminobutyric acid (GABA), are potential targets for drugs with anticonvulsant properties (3). Therefore, an understanding of the structure and function of neurotransmitter transporters may lead to a better understanding of synaptic regulation and could also provide a rational basis for the development of additional, more-specific anti-transporter drugs.

The recent isolation of cDNA clones encoding transporters for GABA (4), the monoamines norepinephrine, dopamine, and serotonin (5–10), and a nonneurotransmitter, betaine (11), has revealed that these carriers comprise a superfamily of homologous proteins. Predictions of membrane topology suggest that these transporters all contain ≈12 membrane-spanning domains, a structural motif shared by a number of carriers that, however, share little mutual sequence homology. The amino acid-sequence identity between the known members of the neurotransmitter transporter superfamily

ranges from ≈44% for the GABA transporter (GAT1) versus the norepinephrine transporter (NET1) to ≈64% for NET1 versus the dopamine transporter (DAT1), indicating that subfamilies exist within the superfamily. In addition to an overall homology, members of this superfamily contain several regions of sequence identity distributed throughout the length of the protein. These regions can be used to design PCR primers which can be employed to clone additional members of the superfamily from brain and other tissues. In this paper we report the results of such a cloning approach, which has yielded a rat brain cDNA[‡] encoding a sodium- and chloride-dependent high-affinity glycine transporter.

MATERIALS AND METHODS

RNA Isolation and PCR. Total RNA was prepared from C6 glioma cells and rat tissues by the acid guanidinium/phenol method (12). cDNA was synthesized directly from 5 μ g of total RNA or mRNA and amplified by the PCR and standard protocols. The primers chosen for amplification consisted of a 128-fold degenerate sense primer corresponding to bases 375–394 of GAT1 with the sequence (5' → 3') GAG CTC GTC GAC AAG AAC GG(TCAG) GG(TCAG) GG(TCAG) GC(TC) TT and a 48-fold degenerate antisense primer corresponding to bases 1108–1030 of GAT1 with the sequence (5' → 3') GGG CCC TCT AGA AA (GA)AT CTG (GA)GT (GAT)GC (GA)GC (AG)TC. The upstream primer corresponds in the GAT1 protein to amino acids 76–82 with the sequence KNGGGAF, and the downstream primer corresponds to amino acids 287–293 with the sequence DAATQIF. With these primers the PCR will generate a 677-base-pair (bp) product from GAT1 mRNA and a 710-bp product from NET1 mRNA. The amplification protocol consisted of a 1-min denaturation at 94°C, a 4-min annealing at 55°C, and a 2-min extension at 72°C for 25–30 cycles on a Perkin–Elmer DNA thermal cycler. A sample of the amplification reaction was analyzed by agarose gel electrophoresis, and the appropriate band was gel-purified and subcloned into pBluescript SK(–).

RNA Blot. Two micrograms of mRNA was subjected to agarose gel/formaldehyde electrophoresis and transferred to filter paper. The blot was hybridized overnight at 42°C under high-stringency conditions [50% formamide/6× standard saline phosphate/EDTA (SSPE; 1× SSPE = 0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/5× Denhardt's solution/salmon sperm DNA at 100 μ g/ml]. The final stringency wash was done in 0.2× standard saline citrate at 65°C for 15 min. The filter was exposed to film for 2–4 days with two intensifying screens at –70°C.

cDNA Isolation and Sequencing. A λ ZAP rat brain cDNA library (13) containing inserts in the 2–4 kilobase-pair (kbp)

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Abbreviation: GABA, γ -aminobutyric acid.

[‡]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M95413).

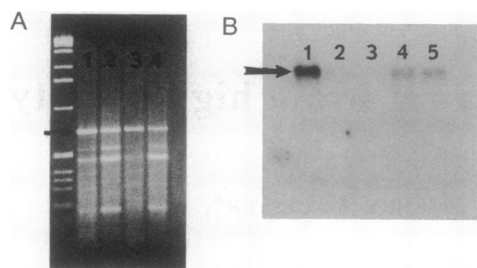


FIG. 1. (A) Ethidium bromide-stained agarose gel of the DNA products generated with described primers and C6 glioma cell cDNA as template. Arrow denotes a major product of ≈ 700 bp. Lanes: 1 and 2, products with C6 glioma cell mRNA as starting material; 3 and 4, products with C6 glioma cell total RNA; 2 and 4, products with a 2-fold dilution of the respective cDNA mixtures. (B) RNA blot of mRNA from C6 glioma cells (lane 1), rat liver and kidney (lanes 2 and 3), and rat forebrain and cerebellum (lanes 4 and 5) probed with the ^{32}P -labeled 700-bp PCR product from C6 glioma cells. Arrow denotes a single transcript of ≈ 3.3 kb. A faint band, visible in lane 2 (liver mRNA) of the original autoradiogram, is difficult to see in this photograph.

size range was screened with the same random primer-labeled PCR product used to probe the RNA blot and under the same hybridization conditions. Six positive clones were identified. The phagemids were rescued from each clone and used for further analysis. Dideoxynucleotide DNA sequencing was done by a combination of nested deletions and primer walks.

Xenopus Oocyte Expression and Transport Assays. *Xenopus* oocytes were defolliculated and maintained in ND96 medium/5% horse serum (14). mRNA was synthesized from 5 μg of plasmid template and gel-purified on a Sephadex G-50 spin column. Ten nanograms of mRNA was microinjected into each oocyte, and the samples were allowed to translate for 4–7 days. Controls consisted of oocytes injected with 50 nl of water. [^3H]Glycine and drugs were added to a total volume

of 50 μl , and uptake assays were done as described (5). Each data point represents the mean of three to five oocytes.

In Situ Hybridization. Rat brain and cervical spinal cord were fixed with 4% paraformaldehyde and processed as described (15). Twenty-five- to 30- μm frozen sections were prepared in both sagittal and horizontal planes and were incubated, free-floating, with ^{35}S -labeled antisense RNA probes. Control sections were probed with ^{35}S -labeled sense RNA to determine nonspecific hybridization.

Sequence Analysis. DNA and amino acid sequences were analyzed on a personal computer with the program PCGENE and on a DEC VAX computer with the Genetics Computer Group package. Data bases were searched on the National Center for Biotechnology Information computer with the program BLAST (16).

RESULTS AND DISCUSSION

Cloning Strategy. C6 glioma cells have been reported to possess transporters for a variety of neurotransmitters, including GABA (17, 18), aspartate (19), glutamate (19), and glycine (20, 21). We, therefore, chose these cells as a potential source of additional members of the neurotransmitter-transporter superfamily. When RNA from C6 cells is amplified by the PCR with the degenerate primers described in *Materials and Methods*, a major product of ≈ 700 bp is seen (Fig. 1A). The nucleotide sequence of this PCR product encodes a peptide with significant homology to both GAT1 and NET1 proteins (data not shown). RNA blot analysis with the PCR product as a probe reveals a single transcript of ≈ 3.3 kilobases (kb) in C6 cell mRNA and in rat forebrain and cerebellum mRNA (Fig. 1B). There is a barely detectable signal in rat liver mRNA and no detectable signal in kidney mRNA. This result indicated that the gene corresponding to this PCR product is expressed in brain but is not expressed (or is expressed at low levels) in nonneural tissue. Therefore, a size-selected λZAP rat brain cDNA library was screened with the C6 cell PCR product, and six positive clones were

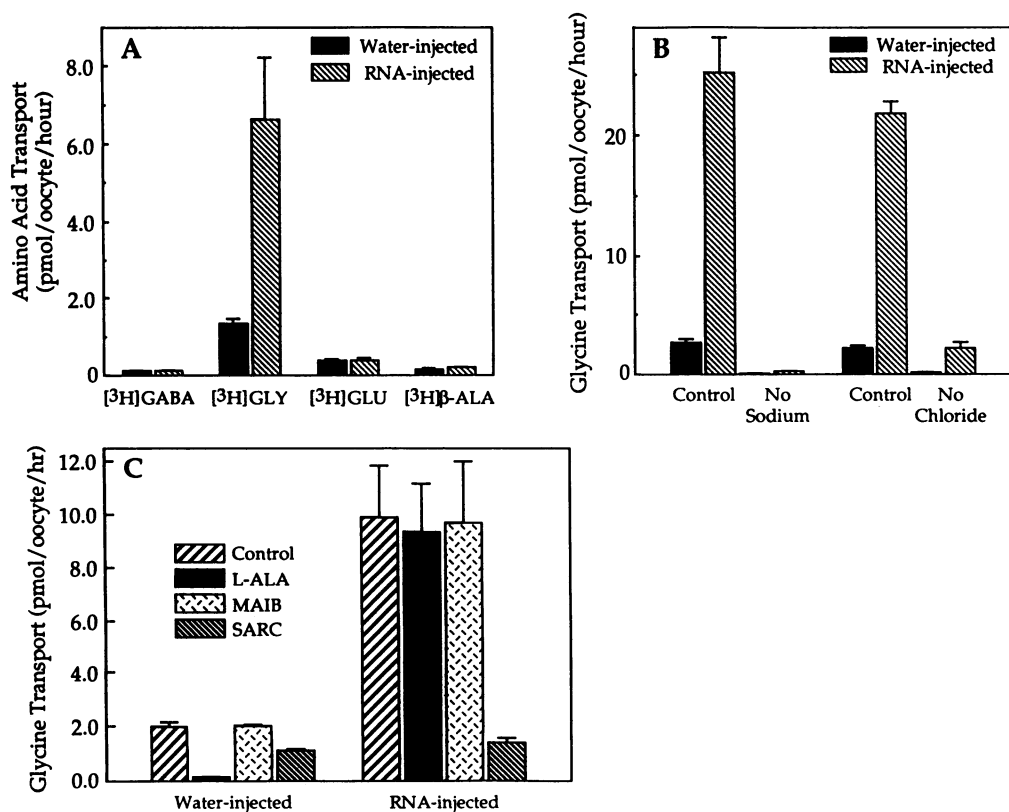


FIG. 2. Expression of GLYT1 in *Xenopus* oocytes. Assays were done 4 days after injection of oocytes with 10 ng of mRNA or 50 nl of water. Each point represents the mean \pm SEM of [^3H]glycine uptake by three to five oocytes. All assays were done at room temperature for 1 hr. (A) Uptake of [^3H]GABA, [^3H]glycine, [^3H]glutamic acid, and β -[^3H]alanine. Each amino acid was used at a final concentration of 1 μM . (B) Uptake of [^3H]glycine in normal saline and saline in which sodium was replaced by lithium (No Sodium) or saline in which chloride was replaced by acetate (No Chloride). (C) Inhibition of [^3H]glycine uptake by three amino acids or amino acid analogs. Unlabeled L-alanine (L-ALA), methylaminobutyric acid (MAIB), or sarcosine (SARC) was included in the incubation mixture at a final concentration of 500 μM .

plaque-purified. Partial nucleotide sequences from these clones indicated that five of the six clones were clearly related to each other and to the original PCR product, whereas the sixth represented an unrelated clone. One of these five clones (clone 9-1B) appeared to be full-length at the 5' end of the open reading frame, and this clone was chosen for further analysis and expression.

Functional Expression. Oocytes injected with 9-1B mRNA were tested for their ability to take up radiolabeled neurotransmitters or related compounds. Fig. 2A shows that oocytes expressing clone 9-1B do not significantly accumulate [3 H]GABA, [3 H]glutamate, or β -[3 H]alanine above background levels. In contrast, the accumulation of [3 H]glycine is enhanced markedly in oocytes injected with 9-1B RNA, reaching levels of ≈ 10 – 20 pmol after 1 hr of incubation in 2.5 μ M [3 H]glycine. This result indicated that clone 9-1B encodes a glycine transporter, and it was designated GLYT1.

Uptake by oocytes injected with GLYT1 mRNA depends on both extracellular sodium and extracellular chloride, indicating that the transporter encoded by this clone mediates ion-coupled active transport of [3 H]glycine (Fig. 2B). Interestingly, the moderate level of [3 H]glycine uptake by control oocytes is also sodium- and chloride-dependent, indicating that *Xenopus* oocytes possess an endogenous transport system for glycine. This fact is not surprising because uptake systems for metabolically important amino acids are found in both neural and nonneural tissues (22). Oocytes injected with GLYT1 mRNA take up [3 H]glycine by a high-affinity mechanism (Fig. 3A and B). Kinetic analysis revealed a K_m of 33 ± 8 μ M (mean \pm SEM, $n = 3$), which is similar to the K_m values reported previously for high-affinity glycine uptake in the central nervous system (23–25). This K_m value is almost 3-fold lower than that reported for [3 H]glycine uptake by C6 glioma cells ($K_m = 95$ μ M) (21). The V_{max} value for the cloned transporter varied, depending on the batch of oocytes, from 120 to 390 pmol per oocyte per hr (mean = 246 ± 78). The oocyte glycine transporter also appears to be a high-affinity transporter with a K_m of ≈ 20 μ M and a V_{max} of ≈ 20 pmol per oocyte per hr (data not shown).

At least three uptake systems able to transport glycine, termed system Gly, system ASC, and system A, have been demonstrated in a variety of tissues and cell types by using amino acids and amino acid analogs as substrates and competitive inhibitors (26). Therefore, the substrate specificity of GLYT1 was examined by using several of the subtype-specific compounds (at a final concentration of 500 μ M) as competitive inhibitors of [3 H]glycine uptake by mRNA-injected oocytes (Fig. 2C). Sarcosine (*N*-methylglycine), a system Gly substrate, inhibits [3 H]glycine uptake by oocytes injected with GLYT1 mRNA by $>85\%$. By contrast, L-alanine, a system ASC substrate, and methylaminoisobutyric acid, a system A substrate, are almost completely ineffective as inhibitors. This inhibition profile suggests that GLYT1 represents a system Gly-like transporter, in agreement with the substrate specificity defined for the native glycine transporter expressed by C6 cells (21). However, the substrate specificity of GLYT1 differs from that described in earlier work on the rat spinal cord (23), where sarcosine was reported to have no significant inhibitory effect at concentrations as high as 1 mM. Thus, GLYT1 may represent one of several glycine-transporter subtypes expressed in brain.

The oocyte endogenous glycine transporter shows an inhibition profile different from GLYT1. The former is inhibited almost 90% by L-alanine (Fig. 2C), L-serine, and L-cysteine (data not shown). This transporter is not blocked by methylaminoisobutyric acid and is only partially inhibited by sarcosine (Fig. 2C). Therefore, the endogenous transporter appears similar to system ASC.

Sequence Analysis. Nucleotide sequencing of the GLYT1 cDNA revealed an open reading frame encoding a protein of

633 amino acids with a molecular mass of 70,570 Da (Fig. 4). The protein contains four putative N-glycosylation sites located in a region, which, for GAT1, is proposed to form a large extracellular loop (4). There are five putative protein kinase C phosphorylation sites. A Kyte–Doolittle analysis of GLYT1 yielded a hydrophobicity profile almost identical to that seen for GAT1 (4) and other members of this transporter superfamily, suggesting that the membrane topology of GLYT1 is similar to proposed models (4, 5, 8). Further analysis with CLUSTAL and PALIGN (PCGENE) showed that GLYT1 is homologous to all known members of the neurotransmitter-transporter superfamily, with identities ranging from 37% to 41% and overall homologies (identity plus similarity) of 53% to 61%. A search of the GenBank and Protein Identification Resource data bases with the program BLAST did not reveal significant homologies between GLYT1 and any cloned genes other than the known members of this superfamily.

Cellular Localization. *In situ* hybridization was used to study distribution of GLYT1 mRNA in the rat central nervous system (Fig. 5A–D). There is prominent labeling in all lamina of the cervical spinal cord, the brainstem nuclei

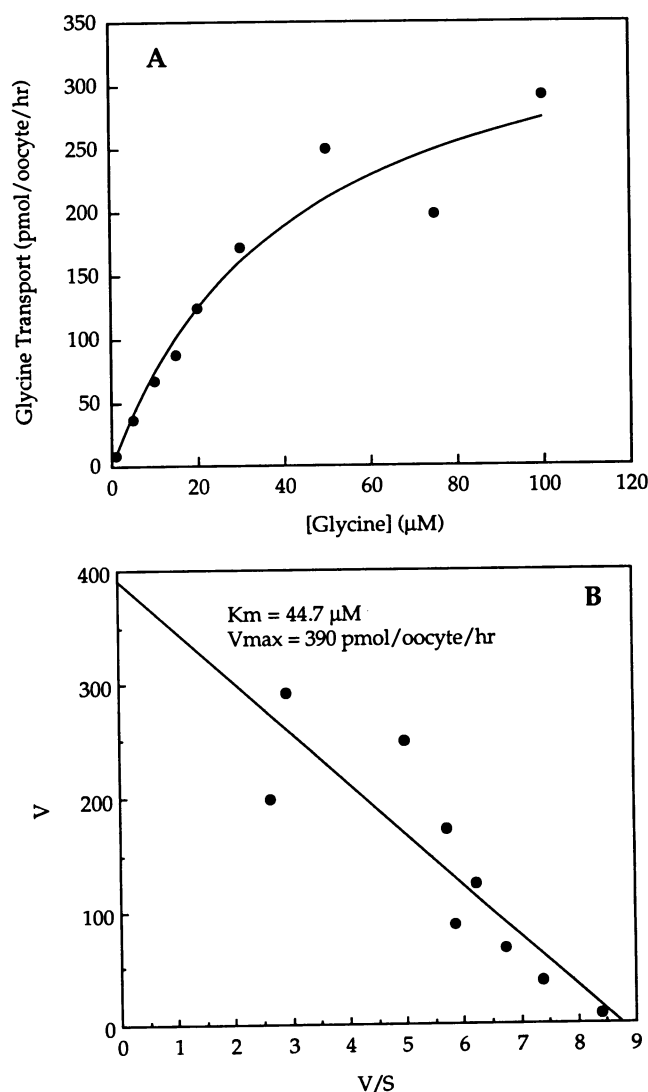
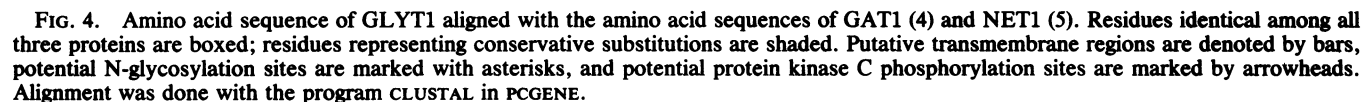


FIG. 3. Kinetics of [3 H]glycine uptake. Michaelis-Menten (A) and Eadie-Hofstee (B) plots of [3 H]glycine uptake by oocytes injected with GLYT1. V, velocity; V/S, velocity/substrate. These values represent the difference between [3 H]glycine uptake by mRNA-injected oocytes and water-injected control oocytes.



Labeling in the brain stem (Fig. 5A) and cervical spinal cord is clearly associated with neuronal cell bodies, although hy-

bridization to glial cells cannot be ruled out. Cerebellar labeling is confined to the Purkinje cell layer, where the silver grains form a continuous band (Fig. 5B). The Purkinje cells themselves are not labeled, and labeling does not appear to be associated with basket, Golgi, or Lugaro cells, which may be located near the Purkinje cell layer. Instead, the labeling pattern indicates that GLYT1 is expressed in Bergmann glia, which are located in this layer. The absence of GLYT1 mRNA in the cerebellar Golgi neurons contrasts with previous reports that, in the rat, these neurons take up [³H]glycine (27) and contain glycine immunoreactivity (28, 29); thus, this finding provides further evidence for the possibility of multiple glycine-transporter subtypes. In the olfactory bulb, there is prominent labeling of many cells located in the glomerular layer (Fig. 5C). Labeled cells also occur in the external plexiform layer, the mitral cell body layer, and the granule cell

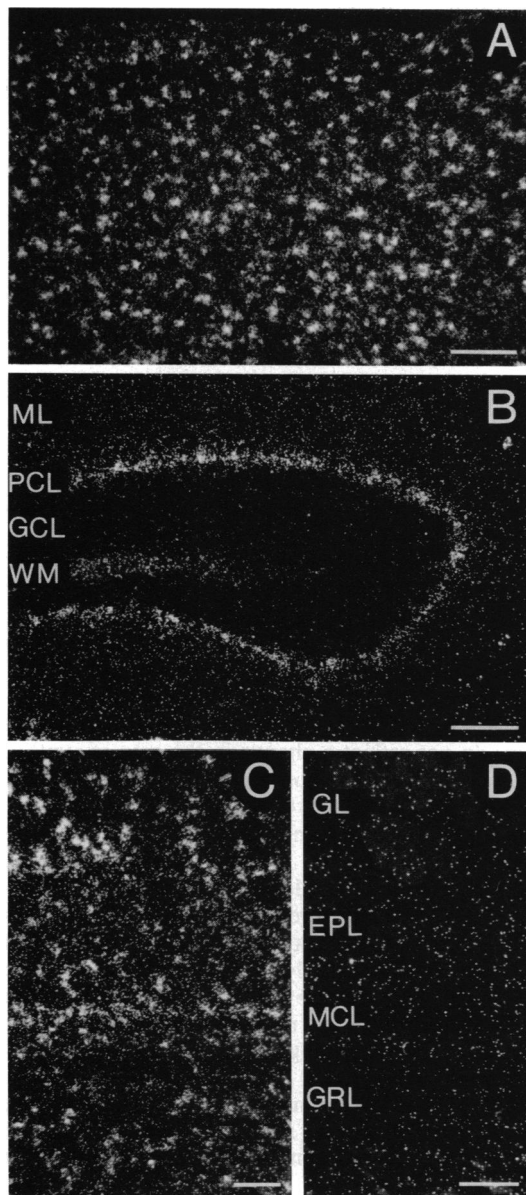


FIG. 5. Localization of GLYT1 expression in the rat central nervous system. (A) Sagittal section through the medulla; numerous heavily labeled cells appear in the spinal trigeminal nucleus. (B) Horizontal section through cerebellum showing a band of labeling in the Purkinje cell layer. ML, molecular cell layer; PCL, Purkinje cell layer; GCL, granule cell layer; WM, white matter. (C) Horizontal section through the main olfactory bulb. (D) Control section. Same as C, except section was incubated with sense mRNA probe. GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GRL, granule cell layer. (Bar = 200 μ m for A and 100 μ m for B–D.)

layer (Fig. 5C). Although localization of [3 H]glycine uptake in the olfactory bulb is equivocal (30), recent immunocytochemical studies suggest that the periglomerular neurons and some cells in the granule cell layer contain glycine (29). As in the other labeled regions, we cannot rule out the possibility that some glia cells in the olfactory bulb express GLYT1.

Availability of the GLYT1 cDNA should facilitate our understanding of glycinergic metabolism in the central nervous system. In addition, further *in situ* hybridization studies of GLYT1 expression will undoubtedly aid in identifying putative glycinergic neurons in the spinal cord, the brainstem, and in forebrain structures not conventionally associated with glycinergic neurotransmission.

Note Added in Proof. After this paper was submitted, Smith *et al.* (31) reported the isolation of a rat brain glycine transporter cDNA clone whose sequence and distribution is similar, but not identical, to that of GLYT1.

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